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2) Becerril, Baltazar; Poul, Marie-Alix; ***Marks, James D. (1)*** ., Biochemical and Biophysical Research Communications, (Feb. 16, 1999) Vol. 255, No. 2, pp. 386-393. ISSN: 0006-291X.

3) Poul, Marie-Alix; ***Marks, James D. (1)*** ., Journal of Molecular Biology, (April 30, 1999) Vol. 288, No. 2, pp. 203-211.
ISSN: 0022-2836.

4) Marks, Cara; ***Marks, James D. (1)., New England Journal of Medicine, (1996) Vol. 335, No. 10, pp. 730-733.
ISSN: 0028-4793.

5) Schier, Robert (1); Adams, G.; Marshall, K.; McCall, A.; Weiner, L.; Bookman, M.; ***Marks, James D., Immunotechnology (Amsterdam), (1996) Vol. 2, No. 4, pp. 290-291.
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ISSN: 1380-2933.

6) Schier, Robert; ***Marks, James D. (1)., Human Antibodies and Hybridomas, (1996) Vol. 7, No. 3, pp. 97-105.
ISSN: 0956-960X.

6) Schier, Robert; ***Marks, James D. (1)*** ; Wolf, Ellen J.; Apell, Gerald; Wong, Cindy; McCartney, John E.; Bookman, Michael A.; Huston, James S.; Houston, L. L.; Weiner, Louis M.; Adams, Gregory P., Immunotechnology (Amsterdam), (1995) Vol. 1, No. 1, pp. 73-81.

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In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library

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Abstract

Background: Antibody-based reagents have failed to live up to their anticipated role as highly specific targeting agents for cancer therapy. Targeting with human single-chain Fv (sFv) molecules may overcome some of the limitations of murine IgG, but are difficult to produce with conventional hybridoma technology. Alternatively, phage display of antibody gene repertoires can be used to produce human sFv. *Objectives*: To isolate and characterize human single chain Fvs which bind to c-erbB-2, an oncogene product overexpressed by 30–50% of breast carcinomas and other adenocarcinomas. *Study design*: A non-immune human single-chain Fv phage antibody library was selected on human c-erbB extracellular domain and sFv characterized with respect to affinity, binding kinetics, and in vivo pharmacokinetics in tumor-bearing scid mice. *Results*: A human single-chain Fv (C6.5) was isolated which binds specifically to c-erbB-2. C6.5 is entirely human in sequence, expresses at high level as native protein in *E. coli*, and is easily purified in high yield in two steps. C6.5 binds to immobilized c-erbB-2 extracellular domain with a K_d of 1.6×10^{-8} M and to c-erbB-2 on SK-OV-3 cells with a K_d of 2.0×10^{-8} M, an affinity that is similar to sFv produced against the same antigen from hybridomas. Biodistribution studies demonstrate 1.47% injected dose/g tumor 24 h after injection of ^{125}I-C6.5 into scid mice bearing SK-OV-3 tumors. Tumor:normal organ ratios range from 8.9:1 for kidney to 283:1 for muscle. *Conclusions*: These results are the first in vivo biodistribution studies using an sFv isolated from a non-immune human repertoire and confirm the specificity of sFv produced in this manner. The use of phage display to

Abbreviations: sFv, single-chain Fv; IgG, immunoglobulin G; V_H, immunoglobulin heavy chain variable region; V_L, immunoglobulin light chain variable region; ECD, extracellualr domain; PBS, phosphate-buffered saline; IPTG, isopropyl-β-D-thiogalactopyranoside; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); IMAC, immobilized metal affinity chromatography; HBS, hepes-buffered saline; CT, chloramine T; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; %ID/g, percentage of injected dose per g of tissue; T:O ratio, tumor:normal organ ratio.

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produce C6.5 mutants with higher affinity and slower k_{off} would permit rigorous evaluation of the role of antibody affinity and binding kinetics in tumor targeting, and could result in the production of a therapeutically useful targeting protein for radioimmunotherapy and other applications.

Keywords: c-erbB-2; sFv; Phage antibody library; Immunotherapy

1. Introduction

With the exception of a few limited applications [1], antibody-based reagents have failed to live up to their anticipated role as highly specific targeting agents for cancer therapy. This has likely been the result of suboptimal delivery of antibody to tumor, due a number of factors including the physiology of the tumors and the large size of IgG molecules. The development of single-chain Fv (sFv) molecules, which retain the binding specificity of a parent IgG in a 26-kDa molecule, addresses some of these issues [2]. Radiolabelled anti-tumor sFv penetrate deeply into human tumor xenografts in mice and are cleared rapidly from circulation and normal tissue, resulting in highly specific tumor retention by as early as 4 h after administration [3]. sFv have typically been created from the immunoglobulin variable region genes of murine hybridomas and expressed in *E. coli*. Limitations of this approach include potential immunogenicity of murine sFv and the fact that many sFv express poorly, or not at all in *E. coli* [4]. Production of human antibodies by conventional hybridoma technology has proven difficult. Recently, it has proven possible to produce human sFv directly in *E. coli* by expressing large antibody gene repertoires on the surface of bacteriophage, and selecting phage-expressing binding antibodies by affinity chromatography (phage display) (see Ref. [5] and, for a review, Ref. [6]). In this report, we describe the application of this technique to produce a human sFv (C6.5) that binds to c-erbB-2, an oncogene product overexpressed by 30–50% of breast carcinomas and other adenocarcinomas. In vitro affinity and binding kinetics and in vivo pharmacokinetics in tumor-bearing scid mice are described and compared to values previously determined for 741F8 sFv′, an sFv molecule produced from a murine IgG [3].

2. Materials and methods

2.1. Preparation of c-erbB-2 ECD

c-erbB-2 ECD with a Ser-Gly-His_6 C-terminal fusion was expressed from Chinese Hamster Ovary cells and purified by immobilized metal affinity chromatography (IMAC) as previously described [7].

2.2. Phage preparation

Phage were prepared from a phagemid library (3×10^7 members) expressing sFv as pIII fusions on the phage surface [5]. The library was created from a repertoire of sFv genes consisting of human heavy and light chain variable region (V_H and V_L) genes isolated from the peripheral blood lymphocytes of unimmunized human volunteers. To rescue phagemid particles from the library, 50 ml of 2 × TY media containing 100 μg/ml ampicillin and 1% glucose were inoculated with 10^8 bacteria taken from the frozen library glycerol stock. The culture was grown at 37°C with shaking to an $A_{600\,nm}$ of 0.8, 7.0×10^{11} colony-forming units of VCS-M13 (Stratagene) added, and incubation continued at 37°C for 1 h without shaking followed by 1 h with shaking. The cells were pelleted by centrifugation at 4500 × *g* for 10 min, resuspended in 200 ml of 2 × TY media containing 100 μg/ml ampicillin and 25 μg/ml kanamycin and grown overnight at 37°C. Phage particles were purified and concentrated by two polyethylene glycol precipitations and resuspended in PBS (25 mM NaH_2PO_4, 125 mM NaCl, pH 7.0) to approximately 10^{13} transducing units/ml ampicillin resistant clones.

2.3. Selection of binding phage antibodies

Phage-expressing sFv which bound c-erbB-2 were selected by panning the phage library on immobilized c-erbB-2 ECD [5]. Briefly, immuno-

tubes (Nunc, Maxisorb) were coated with 2 ml (100 μg/ml) c-erbB-2 ECD in PBS overnight at 20°C and blocked with 2% milk powder in PBS for 2 h at 37°C. One ml of the phage solution (approximately 10^{13} phage) was added to the tubes and incubated at 20°C with tumbling on an over and under turntable for 2 h. Non-binding phage were eliminated by sequential washing (15 times with PBS containing 0.05% Tween followed by 15 times with PBS). Binding phage were then eluted from the immunotubes by adding 1 ml of 100 mM triethylamine, incubating for 10 min at 20°C, transferring the solution to a new tube, and neutralizing with 0.5 ml 1 M Tris-HCl, pH 7.4. Half of the eluted phage solution was used to infect 10 ml of *E. coli* TG1 [8] grown to an $A_{600\,nm}$ of 0.8–0.9. After incubation for 30 min at 37°C, bacteria were plated on TYE plates containing 100 μg/ml ampicillin and 1% glucose and grown overnight at 37°C. Phage were rescued and concentrated as described above and used for the next selection round. The selection process was repeated for a total of five rounds.

2.4. Screening for binders

After each round of selection, 10 ml of *E. coli* HB2151 [9] ($A_{600\,nm} \sim 0.8$) were infected with 100 μl of the phage eluate in order to prepare soluble sFv. In this strain, the amber codon between the sFv gene and gene III is read as a stop codon and native soluble sFv secreted into the periplasm and media [10]. Single ampicillin-resistant colonies were used to inoculate microtire plate wells containing 150 μl of 2 × TY containing 100 μg/ml ampicillin and 0.1% glucose. The bacteria were grown to an $A_{600\,nm} \sim 1.0$, and sFv expression induced by the addition of IPTG to a final concentration of 1 mM [11]. Bacteria were grown overnight at 30°C, the cells removed by centrifugation, and the supernatant containing sFv used directly.

To screen for binding, 96-well microtiter plates (Falcon 3912) were coated overnight at 4°C with 10 μg/ml c-erbB-2 ECD in PBS, blocked for 2 h at 37°C with 2% milk powder in PBS, and incubated for 1.5 h at 20°C with 50 μl of the *E. coli* supernatant containing sFv. Binding of soluble sFv to antigen was detected with a mouse monoclonal antibody (9E10) which recognizes the C-terminal *myc* peptide tag [12] and peroxidase-conjugated anti-mouse Fc antibody (Sigma) using ABTS as substrate [13]. The reaction was stopped after 30 min with NaF (3.2 mg/ml) and the $A_{405\,nm}$ measured. Unique clones were identified by PCR fingerprinting [5] and DNA sequencing. The specificity of each unique sFv was determined by ELISA performed as described above with wells coated with 10 μg/ml of bovine serum albumin, hen egg white lysozyme, bovine glutamyltranspeptidase, c-erbB-2 ECD, VCS M13 (3.5×10^{12}/ml) and casein (0.5%). For ELISA with biotinylated c-erbB-2 ECD, microtiter plates (Immunolon 4, Dynatech) were coated with 50 μl Immunopure avidin (Pierce; 10 μg/ml in PBS) overnight at 4°C, blocked with 1% bovine serum albumin in PBS for 1 h at 37°C and incubated with 50 μl biotinylated c-erbB-2 extracellular domain (5 μg/ml) for 30 min at 20°C. To prepare biotinylated antigen, 0.2 ml c-erbB-2 ECD (1 mg/ml in PBS) was incubated with 0.5 mM NHS-LC-biotin (Pierce) overnight at 4°C and then purified on a presto desalting column (Pierce).

2.5. Subcloning, expression and purification

To facilitate purification, the C6.5 sFv gene was subcloned into the expression vector pUC119Sfi1/Not1Hismyc [14] which results in the addition of a hexa-histidine tag at the C-terminal end of the sFv. Briefly, pHEN-1 vector DNA containing the C6.5 sFv DNA was prepared by alkaline lysis miniprep, digested with *Nco*I and *Not*I, and the sFv DNA purified on a 1.5% agarose gel. C6.5 sFv DNA was ligated into pUC119Sfi1/Not1Hismyc digested with *Nco*I and *Not*I and the ligation mixture used to transform electrocompetent *E. coli* HB2151. For expression, 200 ml of 2 × TY media containing 100 μg/ml ampicillin and 0.1% glucose was inoculated with *E. coli* HB2151 harboring the C6.5 gene in pUC119Sfi1/Not1Hismyc. The culture was grown at 37°C to an $A_{600\,nm}$ of 0.8, soluble sFv expression induced by the addition of IPTG to a final concentration of 1 mM, and the culture grown at 30°C in a shaker flask overnight. sFv was harvested from the periplasm using the following protocol. Cells were harvested by centrifugation at 4000 × *g* for 15 min, resuspended in

10 ml of ice-cold 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20% sucrose, and incubated on ice for 20 min. The bacteria were pelleted by centrifugation at 6000 × *g* for 15 min. and the 'periplasmic fraction' cleared by centrifugation at 30 000 × *g* for 20 min. The supernatant was dialyzed overnight at 4°C against 8 L of IMAC loading buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole) and then filtered through a 0.2-μm filter.

sFv was purified by IMAC. All steps were performed at 4°C on a Perceptive Biosystems BIOCAD Sprint. A column containing 2 ml of Ni-NTA resin (Qiagen) was washed with 20 ml IMAC column wash buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 250 mM imidazole) and 20 ml of IMAC loading buffer. The periplasmic preparation was loaded onto the column by pump and the column washed sequentially with 50 ml IMAC loading buffer and 50 ml IMAC washing buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 25 mM imidazole). Protein was eluted with 25 ml IMAC elution buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 100 mM imidazole) and 4-ml fractions collected. Protein was detected by absorbance at 280 nm and sFv typically eluted between fractions 6 and 8. To remove dimeric and aggregated sFv, samples were concentrated to a volume < 1 ml in a Centricon 10 (Amicon) and fractionated on a Superdex 75 column using a running buffer of HBS (10 mM Hepes, 150 mM NaCl, pH 7.4). The purity of the final preparation was evaluated by assaying an aliquot by SDS-PAGE. Protein bands were detected by Coomassie staining. The concentration was determined spectrophotometrically, assuming an $A_{280\,nm}$ of 1.0 corresponds to an sFv concentration of 0.7 mg/ml.

2.6. Affinity and kinetic measurements

The K_d of C6.5 and 741F8 sFv′ were determined using surface plasmon resonance in a BIAcore (Pharmacia) and by Scatchard analysis. In a BIAcore flow cell, 1400 resonance units (RU) of c-erbB-2 ECD (25 μg/ml in 10 mM sodium acetate, pH 4.5) were coupled to a CM5 sensor chip [15]. Association and dissociation of C6.5 and 741F8 sFv′ (100–600 nM) were measured under continuous flow of 5 μl/min. k_{on} was determined from a plot of $(\ln(dR/dt))/t$ vs. concentration [16]. k_{off} was determined from the dissociation part of the sensorgram at the highest concentration of sFv analyzed [15]. The K_d of C6.5 was also determined by Scatchard analysis [17]. All assays were performed in triplicate. Briefly, 50 μg of radioiodinated sFv was added to 5×10^6 SK-OV-3 cells in the presence of increasing concentrations of unlabelled sFv from the same preparation. After a 30-min incubation at 20°C, the samples were washed with PBS at 4°C and centrifuged at 500 × *g*. The amount of labelled sFv bound to the cells was determined by counting the pellets in a gamma counter and the K_a and K_d were calculated using the EBDA program (V 2.0, G.A. McPherson, 1983).

2.7. Radiolabelling

The C6.5 sFv was labelled with radioiodine using the CT method [18]. Briefly, 1.0 mg of protein was combined with ^{125}I (14–17 mCi/mg) (Amersham, Arlington Heights, IL), or ^{131}I (9.25 mCi/mg) (DuPont NEN, Wilmington, DE) at an iodine to protein ratio of 1:10. Ten μg of CT (Sigma, St. Louis, MO) was added per 100 μg of protein and the resulting mixture was incubated for 3 min at room temperature. The reaction was quenched by the addition of 10 μg of sodium metabisulfite (Sigma) per 100 μg of protein. Unincorporated radioiodine was separated from the labelled protein by gel filtration using the G-50-80 centrifuged-column method [3]. The final specific activity of the CT labelling was 1.4 mCi/mg for the ^{131}I-C6.5 sFv and typically about 1.0 mCi/mg for the ^{125}I-C6.5 sFv.

2.8. Quality control

The quality of the radiopharmaceuticals was evaluated by HPLC, SDS-PAGE, and a live cell binding assay as previously described [3]. The HPLC elution profiles from a Spherogel TSK-3000 molecular sieving column consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. Greater than 98% of the non-reduced ^{125}I-C6.5 sFv preparations migrated on SDS-PAGE as approximately 26 K_d proteins, while the remaining

activity migrated as a dimer. The immunoreactivity of the radiopharmaceuticals was determined in a live cell binding assay utilizing c-erbB-2 overexpressing SK-OV-3 cells (#HTB 77; American Type Culture Collection, Rockville, MD) and c-erbB-2 negative CEM cells (#119; American Type Culture Collection) [3]. Live cell binding assays revealed 49% of the activity associated with the positive cell pellet and less than 3% bound to the negative control cells; these results were lower than those typically seen with 741F8 sFv (60–80% bound) [3].

2.9. Cell surface dissociation studies

Cell surface retention of biotinylated forms of the sFv molecules were measured by incubating 2 μg of either sFv with 2×10^6 SK-BR-3 cells (#HTB 30,; American Type Culture Collection) in triplicate in 20 ml of FACS buffer, with 0.01% azide for 15 min at 4°C. The cells were washed twice with FACS buffer (4°C) and resuspended in 2 ml of FACS buffer; 0.5 ml of the cell suspension were removed and placed in three separate tubes for incubations under differing conditions; 0 min at 4°C, 15 min at 37°C, and 30 min at 37°C. After the incubations, the cells were centrifuged at 500 × *g*, the supernatants were removed, the cell pellets were washed twice (4°C) and the degree of retention of sFv on the cell surface at 37°C (for 15 or 30 min) was compared to retention at 0 min at 4°C.

2.10. Biodistribution and radioimmunoimaging studies

Four- to six-week-old C.B17/Icr-scid mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3 cells (2.5×10^6) in log phase were implanted s.c. on the abdomens of the mice. After about 7 weeks the tumors had achieved sizes of 100–200 mg and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Three days later, biodistribution studies were initiated. ^{125}I-C6.5 sFv was diluted in PBS to a concentration of 0.2 mg/ml and each mouse was given 100 μl, containing 20 μg of radiopharmaceutical, by tail vein injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model #2007, Canaberra, Meridian, CT). Blood samples and whole body counts of the mice were obtained at regular intervals. Groups of eight mice were sacrificed at 24 h after injection and the tumors and organs removed, weighed and counted in a gamma counter to determine the %ID/g [3,19]. The mean and standard error of the mean (SEM) for each group of data were calculated, and T:O ratios determined. Significance levels were determined using Student's *t*-test.

For the radioimmunoimaging studies, tumor-bearing scid mice were injected with 100 μg (100 μl) of ^{131}I-C6.5. At 24 h after injection, the mice were euthanized by asphyxiation with CO_2 and images were acquired on a Prism 2000XP gamma camera (Picker, Highland Heights, OH 44142). Preset acquisitions of 100k counts were used.

3. Results

After four rounds of selection, 9/190 clones analyzed by ELISA expressed sFv which bound c-erbB-2 ECD (ELISA signals greater than 0.4, six-times higher than background). After five rounds of selection, 33/190 clones expressed c-erbB-2 binding sFv. PCR fingerprinting of the 42 positive clones identified two unique restriction patterns, and DNA sequencing of six clones from each pattern revealed two unique human sFv sequences, C4.1 and C6.5 (Table 1). The V_H gene of C6.5 is from the human V_H5 gene family, and the V_L gene from the human Vλ1 family (Table 1). The V_L gene appears to be derived from two different germline genes (HUMLV122 and DPL 5) suggesting the occurence of PCR crossover (Table 1). The V_H gene of C4.1 is from the human V_H3 family, and the V_L gene from the human Vλ3 family (Table 1). C4.1 and C6.5 both bound c-erbB-2 specifically, as determined by ELISA against the relevant antigen and a panel of irrelevant antigens. However, when biotinylated c-erbB-2 ECD was bound to avidin-coated plates and used in ELISA assays, the signal obtained with C6.5 was six-times higher than observed when c-erbB-2 ECD was absorbed to polystyrene (1.5 vs. 0.25). In contrast, C4.1 was not capable of binding to biotinylated c-erbB-2 ECD captured on avidin microtitre plates. Additionally, biotinylated and iodinated C6.5, but

Table 1
Deduced amino acid sequence of C4.1 and C6.5 heavy and light chain. Sequences are aligned to the most homologous human germline gene. Dashes indicate sequence identity, GL = germline gene sequence. DP58 and DP73 [22], IGLV3S1 [23], HUMLV122 AND DPL 5 [24]

Heavy Chains

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	QVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYEMN	WVRQAPGKGLEWVS	YISSSGSTIYYADSVKG	RFTISRDNARNSLYLQMNSLRAEDTAVYYCAR	DLGGYSYGYVGLDY	WGQGTLVTVSS
DP58	E---------- ---------- ----	-- --	----------	- ..--- ---- -----	------ ---- ---------- --- ------		
C6.5	QVQLLQSGAELKKPGESLKISCKGSGYSFT	SYWIA	WVRQMPGKGLEYMG	LIYPGDSDTKYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR	HDVGYCSSSNCAKWPEYFQH	WGQGTLVTVSS
DP73	E---V------V----------	----G	-- - --W--	I------ ---R-------	---- --A---I------- -- A -T-M-Y---		

Light chains

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
C4.1	SELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNIASLTITGAQAEDEADYYC	NSRDSSGNPYWV	FGGGTKVTVLG
IGLV3S1	-S-- - ----------	---- -------	- -------- .---		- - ---T------ - --	--------H V-	
C6.5	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	GHTNRPA	GVPDRFSGSKSGTSASLAISGFRSEDEADYYC	AAWDDSLSG WV	FGGGTKLTVLG
HUMLV122	------ ----------	- - -------	- ----------	DNKK---S	-I-- -------- T-G-T-LQTG--------	GT--S---A	
DPL5	· - A-GT---R---·	---- -S- Y	- -	RNNQ- S	· ---- ---------L------ ---	-- - -	

Table 2
Characterization of anti-c-erbB-2 sFv species

	741F8	C6.5
K_d (BIAcore)	2.6×10^{-8} M	1.6×10^{-8} M
K_d (Scatchard)	5.4×10^{-8} M	2.1×10^{-8} M
k_{on} (BIAcore)	2.4×10^5 $M^{-1}s^{-1}$	4.0×10^5 $M^{-1}s^{-1}$
k_{off} (BIAcore)	6.4×10^{-3} s^{-1}	6.3×10^{-3} s^{-1}
% associated with cell surface at 15 min	32.7	60.6
% associated with cell surface at 30 min	8.6	22.2
%ID/g Tumor	0.8	1.0
T:Blood	14.7	22.9
T:Kidney	2.8	5.6[a]
T:Liver	14.2	22.3
T:Spleen	10.3	34.1
T:Intestine	25.0	29.7
T:Lung	9.4	15.8
T:Stomach	8.9	11.1
T:Muscle	78.8	158.7
T:Bone	30.0	102.7

Characteristics of the murine anti-c-erbB-2 sFv, 741F8, and the human sFv C6.5 are compared. The affinity and dissociation constants were determined by Scatchard plot analysis, unless otherwise stated. Dissociation from c-erbB-2-positive (SK-OV-3) cells was measured in an in vitro live cell assay. The percentage of injected dose per gram (%ID/g) tumor (T) and tumor to organ ratios were determined in biodistribution studies performed in separate groups of scid mice (n = 10–14) bearing SK-OV-3 tumors overexpressing c-erbB-2. SEM are < 35% of the associated values.

[a]Significantly improved, ($P < 0.05$) compared to 741F8 sFv.

not C4.1, bound SK-BR-3 cells overexpressing c-erbB-2. These results indicate that C6.5 binds the native c-erbB-2 expressed on cells, but C4 binds a denatured epitope that appears when the antigen is absorbed to polystyrene.

C6.5 was purified in yields of 10 mg/l of *E. coli* grown in shake flasks and gel filtration analysis indicated a single peak of approximately 27 K_d. The K_d of purified C6.5 was determined using both surface plasmon resonance in a BIAcore and by Scatchard analysis. The K_d determined by BIAcore (1.6×10^{-8} M) agreed closely to the value determined by Scatchard (2.0×10^{-8} M) (Table 2). Kinetic analysis by BIAcore indicated that C6.5 had a rapid on-rate (k_{on} 4.0×10^5 M^{-1} s^{-1}) and a rapid off-rate (k_{off} 6.3×10^{-3} s^{-1})

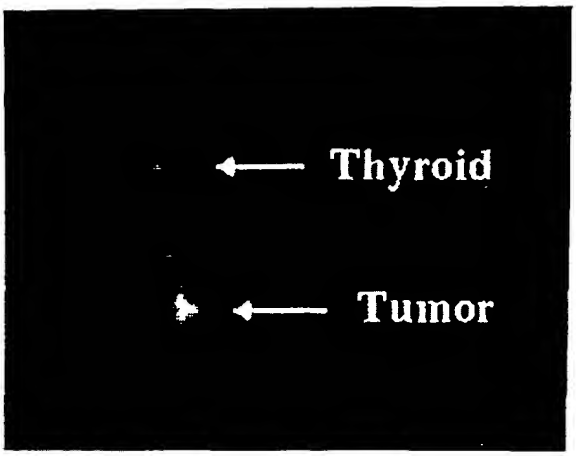

Fig. 1. Radioimmunoimaging of subcutaneous SK-OV-3 tumors in C.B17/ICR-scid mice by ^{131}I-C6.5. Gamma camera images were obtained at 24 h after the i.v. administration of 100 μg (140 μCi) of C6.5. Image acquisition was terminated when 100 000 counts were acquired.

(Table 2). Cell retention assay confirmed that C6.5 dissociated rapidly from the cell surface (Table 2).

After injection of ^{125}I-C6.5 into scid mice bearing SK-OV-3 tumors, 1.47% %ID/g of tumor was retained after 24 h (Table 2). Tumor:normal organ values ranged from 8.9 (tumor:kidney) to 283 (tumor:muscle). These values were higher than values observed for 741F8 sFv′ produced from a murine monoclonal antibody ($K^d = 2.6 \times 10^{-8}$ M). The high T:O ratios resulted in the highly specific visualization of the tumor by gamma scintigraphy using ^{131}I-labelled C6.5 (Fig. 1).

4. Discussion

We have isolated a human sFv from a non-immune phage antibody library which binds specifically to c-erbB-2 in vitro and in vivo. These results are the first in vivo biodistribution studies using an antibody fragment isolated from a non-immune human repertoire, and confirm the specificity of sFv produced in this manner. C6.5 expresses at high level as native protein in *E. coli*, is easily purified in high yield in two steps, and has

an affinity that is similar to sFv produced from hybridomas [3]. The results illustrate potential advantages of this approach compared to producing sFvs from hybridomas. First, the antibodies are entirely human in sequence, and are less likely to be immunogenic than murine sFv. Second, the approach is significantly faster. A single library provides antibodies against most antigens and selections take only 2 weeks to perform. For each hybridoma, however, the V_H and V_L genes have to be successfully isolated and cloned as an sFv DNA construct, a relatively time-consuming process. Once the genes have been successfully cloned, expression levels of different sFv in bacteria vary considerably, and in many instances are too low to produce adequate quantities of protein for characterization and in vivo studies [4]. Even in exceptional cases where very high sFv refolding yields are obtained [20], the final product is a mixture of non-native and native sFv, which are best separated by affinity chromatography. In contrast, sFv produced using phage display are typically expressed at high level in *E. coli* as native protein [5], and are readily purified by a non-functional isolation such as IMAC.

One of the two sFv isolated bound c-erbB-2 immobilized on polystyrene, but not biotinylated c-erbB-2 or c-erbB-2 expressing cells. The result suggests that adsorbtion partially denatures the protein, exposing epitopes that do not exist in solution. Likewise, C6.5 bound biotinylated c-erbB-2 with higher ELISA signal than adsorbed c-erbB-2 and also bound c-erbB-2 expressing cells. Thus, selections performed in solution using biotinylated antigen should optimize the probability that selected sFv will recognize native antigen.

Although C6.5 has an affinity comparable to sFv derived from hybridomas, the k_{off} is relatively rapid, less than 30% of C6.5 remains bound to cell surface c-erbB-2 after 15 min. It should be possible to significantly reduce the k_{off}, and decrease the K_d, by creating and selecting mutant C6.5 phage antibody libraries. We have used this approach to decrease the K_d of a hapten binding human sFv 320-fold, while reducing the k_{off} greater than 100-fold [21]. Production of C6.5 mutants with higher affinity and slower k_{off} would permit rigorous evaluation of the role of antibody affinity and binding kinetics in tumor targeting, and could result in the production of a therapeutically useful targeting protein for radioimmunotherapy and other applications.

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References

[1] Press, O.W., Eary, J.F., Appelbaum, F.R., Martin, P.J., Badger, C.C., Nelp, W.B., Glenn, S., Butchko, G., Fisher, D., Porter, B., Matthews, D.C., Fischer, L.D. and Bernstein, I.D. (1993) N. Engl. J. Med. 329, 1219–1224.

[2] Huston, J.S., Levinson, D., Mudgett, H.M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and Oppermann, H. (1988) Proc. Natl. Acad. Sci. USA 85, 5879–5883.

[3] Adams, G.P., McCartney, J.E., Tai, M.-S., Oppermann, H., Huston, J.S., Stafford, W.F., Bookman, M.A., Fand, I., Houston, L.L. and Weiner, L.M. (1993) Cancer Res. 53, 4026–4034.

[4] Knappik, A., Krebber, C. and Plückthun, A. (1993) Biotechnology 11, 77–83.

[5] Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D. and Winter, G. (1991) J. Mol. Biol. 222, 581–597.

[6] Marks, J.D., Hoogenboom, H.R., Griffiths, A.D. and Winter, G. (1992) J. Biol. Chem. 267, 16007–16010.

[7] Mccartney, J.E., Tai, M.-S., Hudziak, R.M., Adams, G.P., Weiner, L.M., Jin, D., Stafford, W.F., Liu, S., Bookman, M.A., Laminet, A.A., Fand, I., Houston, L.L., Oppermann, H. and Huston, J.S. Prot. Eng. (in press).

[8] Gibson, T.J. (1984) Studies on the Epstein-Barr virus genome. Cambridge University, Ph.D. thesis (see also Ref. 9)

[9] Carter, P., Bedouelle, H. and Winter, G. (1985) Nucleic Acids Res. 13, 4431–4443.

[10] Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P. and Winter, G. (1991) Nucleic Acids Res. 19, 4133–4137.

[11] De Bellis, D. and Schwartz, I. (1990) Nucleic Acids Res. 18, 1311.

[12] Munro, S. and Pelham, H.R.B. (1986) Cell. 46, 291–300.

[13] Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989) Nature 341, 544–546.
[14] Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, R.E., Jones, P.T., Low, N.M., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E. and Winter, G. (1994) EMBO J. 13, 3245–3260.
[15] Johnsson, B., Löfås, S. and Lindqvist, G. (1991) Anal. Biochem. 198, 268–277.
[16] Karlsson, R., Michaelsson, A. and Mattsson, L. (1991) J. Immunol. Methods 145, 229–240.
[17] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660.
[18] DeNardo, S.J., Peng, J.S., DeNardo, G.L., Mills, S.M. and Epstein, A.L. (1986) Nucl. Med. Biol. 13, 303–310.
[19] Adams, G.P., DeNardo, S.J., Amin, A., Kroger, L.A., DeNardo, G.L., Hellstrom, I. and Hellstrom, K.E. (1992) Antibody Immunoconj. Radiopharm. 5, 81–95.
[20] Huston, J.S., Mudgett-Hunter, M., Mei-Sheng, T., McCartney, J., Warren, F., Haber, E. and Opperman, H. (1991) Methods Enzymol. 203, 46–88.
[21] Marks, J.D., Griffiths, A.D., Malmqvist, M., Clackson, T., Bye, J.M. and Winter, G. (1992) Bio/Technology. 10, 779–783.
[22] Tomlinson, I.M., Walter, G., Marks, J.D., Llewelyn, M.B. and Winter, G. (1992) J. Mol. Biol. 227, 776–798.
[23] Fripplat, J.-P., Chuchana, P., Bernard, F., Lefranc, G., Lefranc, M.-P. (1990) Nucleic Acids Res. 18, 7134.
[24] Williams, S. and Winter, G. (1993) Eur. J. Immunol. 23, 1456–1461.